A Simple DNA-Based Screening Approach for the Detection of Crop Species in Processed Food Materials

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Summary

Ensuring that food manufacturers and suppliers are adhering to current EU legislation on labelling is important for preventing food fraud, enabling consumer choice and ensuring that ingredients are listed correctly in the interest of public health. This highlights the requirement for reliable and sensitive methods for detection and identification of ingredients, particularly those which may be allergenic. Molecular biology approaches including the Polymerase Chain Reaction (PCR) have become increasingly common for the detection of DNA targets in food. The aim of the present study was to design and demonstrate proof-of-principle for a simple end-point PCR and capillary electrophoresis (CE) system for the detection of a subset of crop species in processed foods and to demonstrate minimum good practice in the evaluation of such an approach. A system of this kind could be used as a screening approach in Official Control Laboratories.

PCR assays were designed based on taxon-specific gene targets from cotton, lupin, maize, soya and sugar beet. Primers were tested using authenticated positive control material and amplicons analysed using the Agilent 2100 Bioanalyzer. The five assays were optimised for simultaneous application using the same cycling conditions and their fitness for purpose demonstrated by applying them to a range of processed food materials.

The assays for lupin, maize and soya repeatedly amplified DNA from a range of processed food materials where the crop species were present on the ingredient list. The cotton and sugar beet assays showed no cross-reactivity with other crop species and demonstrated no false-positives when applied to the range of processed food materials, only producing a detectable response when in the presence of the target DNA sequence from the positive control. The present study was successful in developing a simple, singleplex lab-on-a-chip approach for the simultaneous detection of common ingredients in foods, the principles of which can be applied to various fields within the area of food authenticity testing.

Keywords

PCR, lab on a chip, screening, maize, soya, cotton, sugar beet, lupin, allergens

Introduction

Food authenticity and testing for food fraud is centred on the correct identification of ingredients as specified on product labels. The authorised sale and distribution of a product can be attributed to a number of factors including the manufacturer's adherence to current legislation on food labelling, the correct composition of the food with a description of its origin, adherence to an appropriate level of quality in production and identification of any fraudulent replacement of more expensive ingredients with cheaper ones. In recent years issues surrounding food authenticity have arisen due to fraudulent use of components not stated on the label. Examples of this include the UK/EU Horse Meat issue in early 2013 where significant levels of horse DNA were detected in products that were labelled as beef[1]. This had a major impact on consumer confidence in food safety and authenticity and led to other findings of meat adulteration including the detection of porcine DNA in meat that was labelled as Halal[2]. The importance of ensuring traceability in the food supply network has recently been highlighted and reinforced through the publication of the Elliott review[3]. Other examples of food adulteration include the addition of inexpensive corn syrup to honey and premium fruit juices being diluted with lower grade products to reduce production costs[4]. The prevalence of food fraud combined with its potential influence on the food industry highlights the need for suitable methods for detection and identification of ingredients within a product, whether these are stated on the label or present as adulterants. This will assist in preventing fraudulent or potentially dangerous products from entering the food chain, thus increasing consumer confidence and supporting food businesses by providing guidance on and confirmation of adherence to current legislation on food labelling.

EU Regulation 1169/2011 on the provision of food information to consumers was adopted in January 2012. This regulation includes new legislation that will apply to all EU member states as of 13th December 2014 introducing significant changes to the existing legislation on food labelling. Some of the significant aspects associated with this regulation include a requirement for the emphasis of certain allergen information on the ingredients of pre-packed foods and provision of such information for non-pre-packed foods (including foods sold in restaurants and cafes). For pre-packed food the primacy of the ingredients list is established as the source of information on allergens rather than an "advisory box" on a product label; the new legislation stipulates that individual allergenic species must be clearly labelled in the ingredients list. In light of these changes it is imperative that Public Analyst and trade laboratories are in a position to utilise appropriate methodologies for the detection of ingredients such as allergens in the event of any legal disputes that arise over food production. Molecular techniques such as the Polymerase Chain Reaction (PCR), real-time PCR and automated electrophoresis have been extensively used and are well characterised for species detection owing to their high specificity and sensitivity, rapid processing times and relatively low associated costs. PCR has also been used for the detection of Genetically Modified Organisms (GMOs), adulterant species, pathogens and allergens in food[5].

There are many methods available for the detection of allergenic proteins in food products, although DNA-based techniques often have advantages in terms of better sensitivity and greater specificity. In addition, as an analyte DNA is generally more stable than proteins

which can become denatured easily during cooking or processing of food[6] (albeit it is possible for DNA to become degraded owing to depurination in highly acidic food systems, such as those containing tomato). Currently more than 17 million people in Europe suffer from food allergies (over 2% of the population)[7], which presents a significant public health challenge that must be met by EU regulators, the food industry and healthcare systems. Symptoms of food allergy can manifest with varying levels of severity from mild swelling and itching to anaphylactic shock which can be fatal. Affected individuals rely heavily on accurate labelling of food, as ingestion of an ingredient to which they are allergic could be life threatening. Specialist food products are available but these are often more expensive and unobtainable for some. Soya and lupin are two allergens that were included in the present study, as listed in Annex IIIa of EU Directive 2003/89 and Annex II to Regulation 1169/2011[8]

The aim of the present study was to design an end-point PCR and capillary electrophoresis (CE) system for the detection of a range of crop species, some of which present known allergen risks in processed foods. The target species included;

- those which are common ingredients in a broad variety of foods (maize)
- those which could demonstrate capability for detecting transgenic species or species that may be used to adulterate food products (sugar beet and cotton)
- those ingredients which are listed allergens (soya and lupin).

A combination of published information and in-house design of PCR primers using NCBI PrimerBLAST[9] along with DNA sequences deposited in GenBank, were used in the design and optimisation of the assays. The assays were tested, optimised and applied to processed food materials to demonstrate proof-of-principle for the method of detection. The project encompassed the use of PCR and Capillary Electrophoresis using an Agilent 2100 Bioanalyzer. This "lab-on-a-chip" approach is a simple, cost effective method of screening a sample using end-point PCR with readily available laboratory instrumentation. Although a range of real-time PCR assays are available and are well-characterised, the proliferation and utility of "lab-on-a-chip" approaches suggest that Public Analyst and trade laboratories will benefit from the dissemination of the knowledge gained in the development of a PCR-CE-based detection system of this kind. The paper also seeks to demonstrate minimum good practice in the evaluation of such an approach.

Materials and Methods

Sample Selection

The species that were selected for inclusion in the present study were soya, maize, cotton, sugar beet and lupin. Authenticated positive controls for soya, maize, cotton, and sugar beet were obtained as dried Certified Reference Materials (CRMs) from IRMM via LGC Standards (UK), details of which can be found in Table 1. An authenticated positive control for lupin was obtained as pre-extracted DNA from the Royal Botanical Gardens (*Lupinus luteus* DNA; Voucher ID ABH31123; DNA bank number 15870; Kew, UK). Authenticated reference materials were also provided for: aubergine, cauliflower, celery, mustard, okra,

potato, oilseed rape and rice, so that cross reactivity studies across a range of common crops could be carried out.

Table 1 - Certified Reference Materials (CRMs) obtained from IRMM for the Study

Crop Type	IRMM Catalogue Number	Variety
Soya	ERM-BF410ak	Roundup Ready™
Maize	ERM-BF413ak	MON810
Cotton	ERM-BF429a	T304-40
Sugar beet	ERM-BF419a	H7-1

In order to establish fitness for purpose of the procedure a range of processed food materials were kindly provided by Waitrose and these are listed in Table 2.

Table 2 - List of Processed Food Materials included in the Study

Food	Ingredients (species of interest to the present study are highlighted in **bold)**	Allergen Information
Trufree digestive biscuits	**Maize starch**, palm oil, sugar, **maize flour**, **soya flour**, **soy bran**, buckwheat flour, **sugar beet syrup**, modified tapioca starch, salt, raising agents.	**Soya**
Trufree custard creams	Vanilla cream: sugar, vegetable fat, whole milk powder, sweet whey protein, whey protein concentrate, emulsifier soya lecithin. **Maize starch**, sugar, **soya flour**, vegetable fat, rice flour, salt, guar gum, raising agents.	Milk, **soya**, **may contain lupin**
Tuc crackers	Wheat flour, vegetable oil, glucose syrup, barley malt extract, salt, raising agents, dried whole egg, flour treatment agent.	Egg, gluten, may contain sesame seeds and milk
Zest cheese bites parmesan	Wheat flour, butter, cheese, dried glucose syrup, parmesan cheese, spice and herb mix, milk powder, raising agents, glaze, colouring.	May contain nuts, peanuts, **soy**, egg, **lupin**
Burgen soya and linseed brown bread	Wheat flour, water, linseed, **soya flour**, cracked wheat, **kibbled soya**, wheat protein, yeast, emulsifiers, flour treatment agent.	Wheat, gluten, **soya**
Waitrose gluten free pittas	Water, tapioca starch, rice flour, potato starch, yeast, rapeseed oil, psyllium husk powder, dextrose monohydrate, raising agents, stabiliser, dried free range egg albumin, humectant, **maize flour**, salt, preservative, sugar, emulsifier.	Egg

Food	Ingredients (species of interest to the present study are highlighted in **bold)**	Allergen Information
Genius brown bread	Water, tapioca starch, rice flour, potato starch, rapeseed oil, **maize starch**, psyllium husk powder, treacle, humectant, dried egg white, yeast, salt, millet flakes, flax seeds, **sugar beet fibre**, sugar syrup, rice starch, preservative, flavourings.	Egg, may contain nuts
Chocolate muffins	Sugar, wheat flour, dark chocolate chips, rapeseed oil, free range egg, water, cocoa powder, milk proteins, cornflour, raising agents, emulsifier. Chocolate chips: Sugar, cocoa mass, cocoa butter, soya lecithin.	Egg, gluten, milk, **soya**
Gluten free date and walnut slices	Sultanas, sugar, vegetable margarine, rice flour, water, chopped dates, walnuts, rapeseed oil, ground almonds, potato starch, pureed dates, dried free range egg, glucose syrup, humectant, **maize flour**, mixed spice, raising agents, stabilisers.	Egg, nuts

DNA Extractions – In-House Validated CTAB Method

DNA was extracted from the CRM's for soya, maize, cotton and sugar beet, as well as the processed food materials, using a modified CTAB (Cethyltrimethylammonium Bromide) method[10]. Samples were incubated at 65°C for at least 12 hours in a mixture of CTAB buffer, β-mercaptoethanol (0.2% final concentration; Sigma), RNase A (100mg/mL; Qiagen), Proteinase K (20mg/mL; Qiagen) and nuclease-free water (Ambion). The DNA-CTAB complexes were stripped of lipids and carbohydrates following several centrifugation steps with Chloroform (Sigma) before being precipitated using CTAB and 1.2M NaCl. DNA pellets were washed with Ethanol (Fisher Scientific) and re-suspended in 75-100µL of nuclease-free water. Extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer. For the lupin positive control material DNA was provided in a pre-extracted format by Kew Gardens, which had been eluted in aqueous Tris-EDTA buffer and purified via a caesium chloride/ethidium bromide gradient; (http://apps.kew.org/dnabank/homepage.html; accessed 24/11/14).

For fresh plant samples such as celery and aubergine tissue disruption was necessary to provide a sufficiently large surface area for DNA extraction. This was achieved by grinding with dry ice in a food processor, where required.

PCR – Primer Design

Existing primers were sourced from the current literature, except for the lupin assay where novel primers were designed using primerBLAST[9] based on sequence data deposited in GenBank. All primers were ordered from Sigma Aldrich (UK). Each of these assays along with sequence information is listed in Table 3.

Table 3 - Primer Sequence Information and Technical Details for each of the Assays included in the Study

Species	Target Gene	Amplicon Size (bp)	Sequence (5' to 3')	Oligo (bp)	Source
Soya	Lectin	123	GCCCTCTACTCCACCCCCATCC	22	11
			GCCCATCTGCAAGCCTTTTTGTG	23	
Maize	Zein	151	CGCCAGAAATCGTTTTTCAT	20	12
			GGTGGTGTCCTTGCTTCCTA	20	
Cotton	Alcohol de-hydrogenase C	73	CACATGACTTAGCCCATCTTTGC	23	13
			CCCACCCTTTTTTGGTTTAGC	21	
Sugar beet	Glutamine Synthetase	121	GACCTCCATATTACTGAAAGGAAG	24	14
			GAGTAATTGCTCCATCCTGTTCA	23	
Lupin	trnL-trnF intergenic spacer region	68	TCTTTTACAAATGGATCTGAGTGGA	25	Present study
			ATTCAAAGACTTGTGTTGTGATTGT	25	

PCR Cycling Conditions

For primer optimisation, primer concentrations were varied across the range of 0.25, 0.50, 0.75 and 1.00µM. The annealing temperature (T_a) was varied across the range of 56, 58, 60 and 62°C.

The final optimised PCR conditions for each reaction used 25ng of control DNA template, 1µM of each of forward and reverse primers (Sigma Aldrich), nuclease-free sterile water, and "AmpliTaq® gold 2X PCR master mix" (Life Technologies) in a final volume of 25µL. Reactions were run on an ABI9700-9 thermal cycler (Life Technologies). The final optimised cycling conditions were standardised at 10mins at 95°C followed by 40 cycles of 1min at 94°C, 1min at an annealing temperature (T_a) of 60°C and 1min at 72°C, with a final elongation step at 72°C for 5mins.

Cross-Reactivity Studies

The five assays for soya, maize, cotton, sugar beet and lupin were tested against DNA from all positive control materials and other crop varieties (inclusive of soya, maize, cotton, sugar

beet, lupin, aubergine, cauliflower, celery, mustard, okra, potato, oilseed rape and rice) to assess assay specificity.

Determination of the LOD of each Assay

The Limit of Detection (LOD) for each assay was established using three separate six-point five-fold dilution series of the positive control DNA template. These ranged from $5x10^4$ to 16pg, 80 to 5pg and 16 to 1pg DNA per PCR, depending on the assay. Cycling conditions were as described above with a T_a of 62°C and a final primer concentration of 0.25µM.

Application of Assays to Processed Food Samples

DNA was extracted using the modified CTAB approach previously described. The assays for cotton, sugar beet, soya, maize and lupin were applied to the DNA extracts using the optimised PCR conditions with primer concentrations of 0.25µM and a T_a of 62°C. Each assay was applied to each sample in duplicate, along with the appropriate positive controls, extraction blanks and no-template controls (NTC's).

Analysis of PCR Products using the Agilent Bioanalyzer 2100

Following all PCR experiments amplicons were run on the Agilent Bioanalyzer 2100 using a DNA1000 kit (Agilent Technologies). DNA chips were set up as instructed in the manufacturer's guidelines and each run analysed using the 2100 Expert Software (Agilent Technologies). Amplicon size was measured against a 25-1000bp molecular weight ladder.

Results

DNA Extractions – In-house Validated CTAB method

DNA was extracted from CRM flours as detailed in Table 4. A total yield over 1µg (10ng/ µL) from the CRMs was considered to be sufficient for downstream PCR experiments.

Table 4 - DNA Yield and Purity Values for Positive Control Materials used in the Study

Species	Mean yield (ng/µL)	Mean A260/A280	Mean A260/A230
Cotton	18.88	1.75	1.31
Sugar beet	30.14	2.10	1.59
Lupin	195.81	0.98	0.88
Soya	58.58	1.63	0.94
Maize	191.29	1.84	2.28

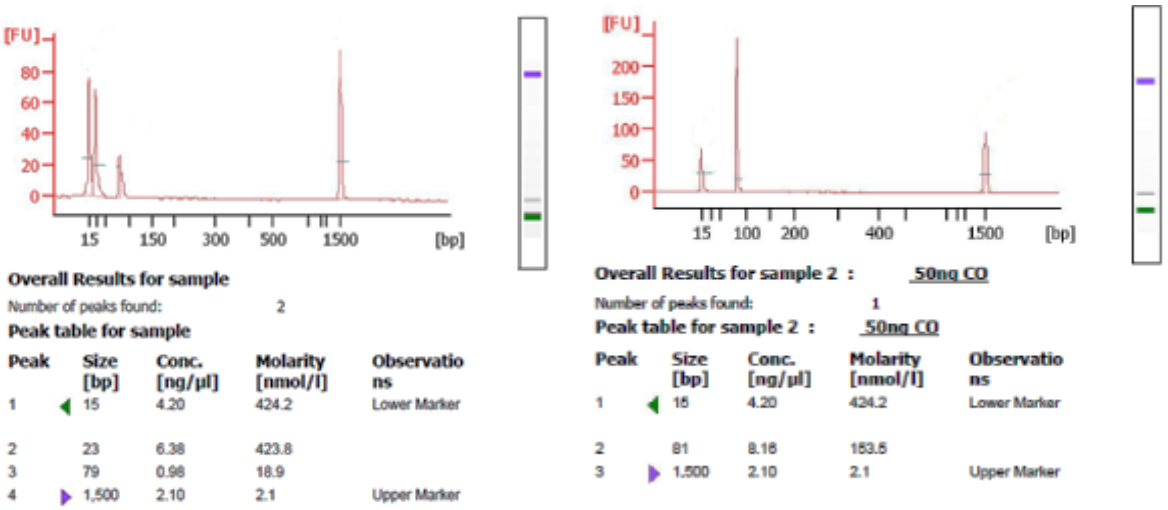
A mean A260/A280 ratio of approximately 1.8 and a mean A260/A230 ratio of approximately 2 represent a "pure" DNA sample. Values were recorded using a NanoDrop ND-1000 spectrophotometer.

PCR Analyses

PCR assays for each species were tested against the relevant positive control material to assess fitness for purpose in detecting the correct target. The target was considered detectable when an amplicon of the appropriate size was confirmed by the 2100 Expert software (Agilent Technologies) that also gave a clear peak of over 20 fluorescent units (FU) visible on the electropherogram.

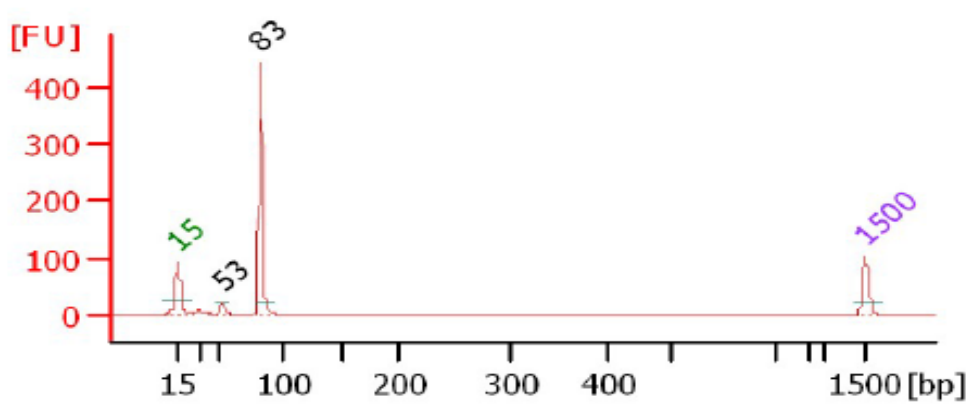
It is worth noting that whilst the lupin assay was successful in amplifying a product at around the predicted size, amplification efficiency appeared relatively poor. This often produced a product which could only be detected at a fluorescence level that was slightly over the software-set threshold of 20FU, as illustrated in figure 1.

Figure 1 - Electropherogram Images to Demonstrate varying Signal Outputs for some Assays



The PCR amplicons generated for each assay are represented by electropherogram graphs on the Agilent 2100 Bioanalyzer, with amplicon size in bp on the x-axis and relative fluorescent units on the y-axis. A "gel-like image", similar in style to that produced by a traditional agarose gel, can be observed on the right of each electropherogram above. The image on the left shows the output for the lupin assay when applied to relevant positive control material, with a peak at 79bp. However, despite being above the calling threshold the concentration of this product is low in relation to the molecular markers at 15 and 1500bp (green and purple, respectively). In comparison, the electropherogram on the right for the cotton assay (applied to a cotton positive control) demonstrates a strong signal over 200FU for an 81bp amplicon which is over double the concentration of the markers.

Figure 2 – Electropherogram Image for Cotton Assay



Overall Results for sample 4 : CO+

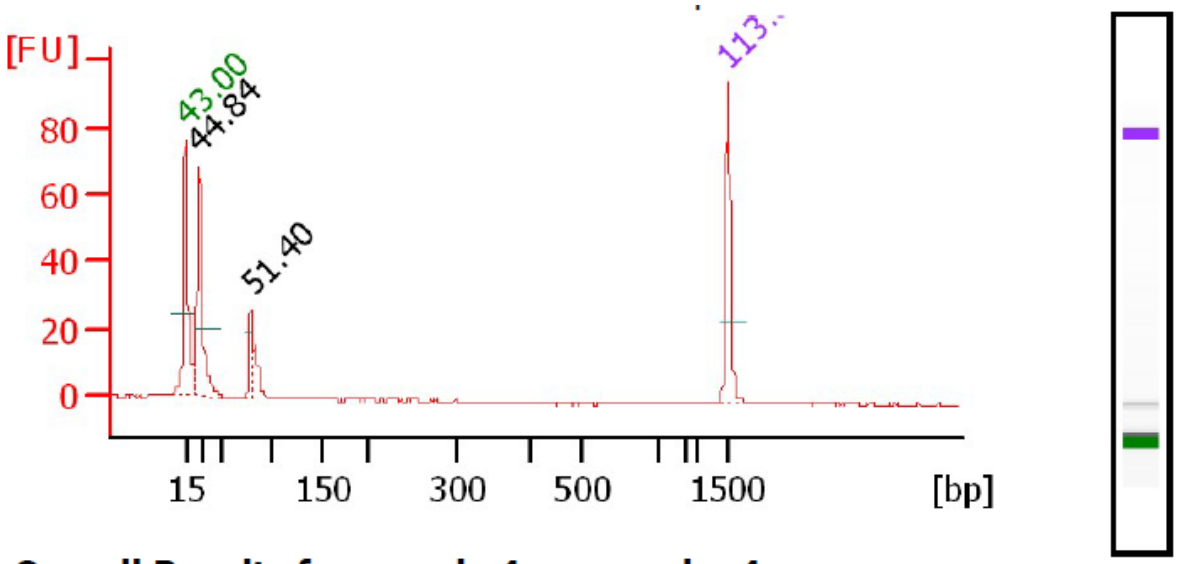
Number of peaks found: 2

Peak table for sample 4 : CO+

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	15	4.20	424.2	Lower Marker
2	53	0.78	22.4	
3	83	12.36	226.1	
4	1,500	2.10	2.1	Upper Marker

As in figure 1, the PCR products generated by each assay are represented by an electropherogram. Figure 2 shows the electropherogram generated by the cotton assay on the cotton positive control. An amplicon can be observed at 83bp (x-axis), which is significantly over the 20FU threshold (y-axis). Molecular markers can be observed at 15 and 1500bp and represented in green and purple, respectively. A product was also detected at 53bp, which may be indicative of the presence of primer dimers. In addition, a "band" is observed on the gel-like image to the right of the figure.

Figure 3 - Electropherogram Image for the Lupin Assay



Overall Results for sample 4 : lup4

Number of peaks found: 2

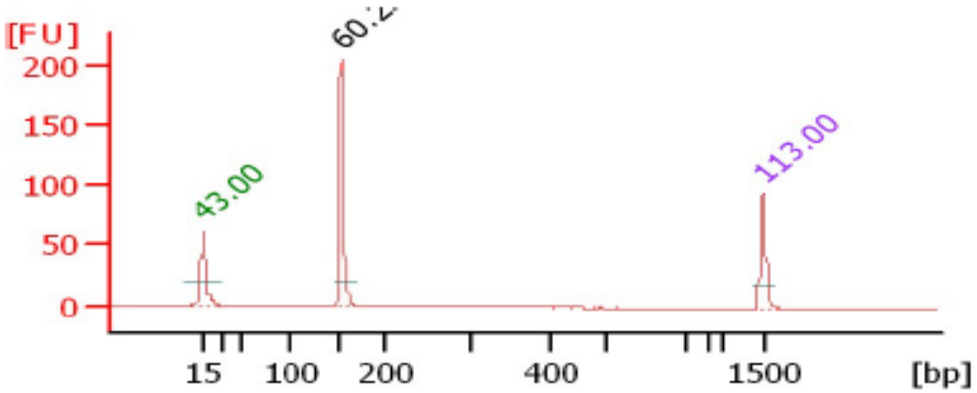
Peak table for sample 4 : lup4

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	15	4.20	424.2	Lower Marker
2	23	6.38	423.8	
3	79	0.98	18.9	
4	1,500	2.10	2.1	Upper Marker

Figure 3 shows the electropherogram generated by the lupin assay on the lupin positive control. An amplicon can be observed at 79bp (x-axis), which is over the 20FU threshold (y-axis). Molecular markers can be observed at 15 and 1500bp and represented in green and purple respectively. A product was also observed at 23bp, which may be due to the detection of the primers used in the assay.

Note that a double peak or "shoulder" can sometimes be observed when using the Agilent 2100 Bioanalyzer which is not necessarily recorded by the software as it is below the default 20FU threshold. Detection of "ghost peaks" with the Agilent 2100 Bioanalyzer has been described at: (http://www.mbl.edu/jbpc/files/2014/05/Bioanalyzer_for_NGS_slideshow.pdf, accessed 09/12/14)

Figure 4 – Electropherogram Image for the Maize Assay



Overall Results for sample 10 : MA0.25

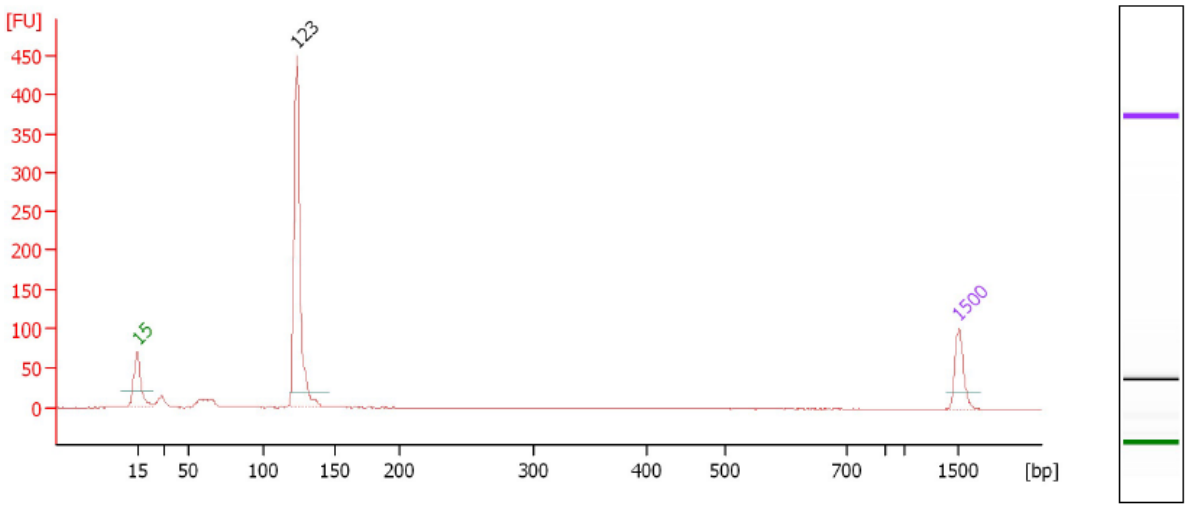
Number of peaks found: 1

Peak table for sample 10 : MA0.25

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	15	4.20	424.2	Lower Marker
2	153	6.50	64.2	
3	1,500	2.10	2.1	Upper Marker

Figure 4 shows the electropherogram generated by the maize assay on the maize positive control. An amplicon can be observed at 153bp (x-axis), which is significantly over the 20FU threshold (y-axis). Molecular markers can be observed at 15 and 1500bp and represented in green and purple, respectively.

Figure 5 - Electropherogram Image for the Soya Assay



Overall Results for sample 12 : SO+

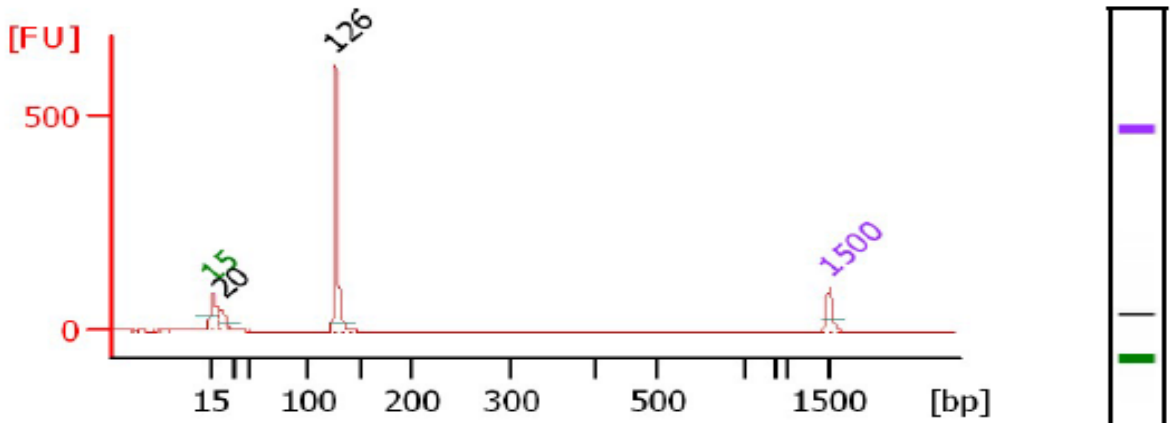
Number of peaks found: 1

Peak table for sample 12 : SO+

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	15	4.20	424.2	Lower Marker
2	123	14.39	176.8	
3	1,500	2.10	2.1	Upper Marker

Figure 5 shows the electropherogram generated by the soya assay on the soya positive control. An amplicon is observed at 123bp (x-axis), which is significantly over the 20FU threshold (y-axis). Molecular markers can be observed at 15 and 1500bp, represented in green and purple respectively. The gel-like image to the right of the figure illustrates the magnitude of the response associated with the "band" representative of the PCR amplicon.

Figure 6 - Electropherogram Image for the Sugar Beet Assay



Overall Results for sample 5 : SU7

Number of peaks found: 2

Peak table for sample 5 : SU7

Peak	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
1	15	4.20	424.2	Lower Marker
2	20	3.22	247.4	
3	126	16.80	201.8	
4	1,500	2.10	2.1	Upper Marker

Figure 6 shows the electropherogram generated by the sugar beet assay on the sugar beet positive control. An amplicon is observed at 126bp (x-axis), which is significantly over the 20FU threshold (y-axis). Molecular markers can be observed at 15 and 1500bp and represented in green and purple, respectively. The gel-like image to the right of the figure demonstrates the magnitude of the response associated with the "band" representative of the PCR amplicon.

Primer Optimisation

Annealing temperature (T_a) and primer concentration were selected as PCR variables for the optimisation studies. T_a was varied between 56, 58, 60 and 62°C and final primer concentrations between 0.25, 0.50, 0.75 and 1.00µM for each primer. Each pairwise temperature-concentration combination was applied to the five assays.

The results showed all of the assays responded well to T_a of 62°C with minimal non-specific amplification. In terms of amplification efficiency all assays produced electropherogram peaks that were over the 20FU threshold with the exception of the lupin assay. However,

comparatively the strongest fluorescence was recorded for the lupin assay at 62°C suggesting that amplification is most efficient at this T_a based on the conditions investigated. It was also noted that a primer concentration of 0.25µM (250nM) was optimal for all five assays. Based on these results the conditions of 62°C T_a and 0.25µM primer concentration were chosen as the optimal PCR conditions for the remainder of the study.

The lupin assay suffered from poor amplification efficiency so attempts were made to improve this by varying the starting amount of DNA template across a 4-point 2-fold dilution series from 12.5 to 100ng per PCR. This appeared to make little difference to the assay although a product of 76bp was still amplified and was detectable above 20FU. Although the assay performed inefficiently it appeared to be fit for purpose based on the repeated amplification of a product from the positive control material with no cross-reactivity.

Cross-Reactivity Studies

All five assays were subject to cross-reactivity studies across the range of authenticated reference materials and additional crop species. If a PCR product was observed that was within ±10bp sizing accuracy from the positive control repeatability studies were undertaken to ensure that any product formed was sufficiently distinguishable from the control amplicon.

Determination of the LOD of Each Assay

Initial experiments to characterise the LOD for each assay involved the dilution of control templates over a six-point five-fold series from $5x10^4$ to 16pg DNA per PCR. All assays were capable of detecting the desired target down to at least 80pg of DNA per PCR with the maize, soya and sugar beet assays working to 16pg. Further experiments were conducted to determine the LOD across a six-point two-fold dilution series ranging from 80 to 5pg DNA per PCR for cotton and lupin and 16 to 1pg per PCR for soya, sugar beet and maize.

Table 5 - LOD Determinations for Each Assay

Assay/Species	LOD (pg DNA per PCR)
Cotton	400
Lupin	400
Maize	8
Soya	4
Sugar beet	2

The above results are based on serial dilutions of the respective positive control material.

The cotton and lupin assays were capable of repeatedly detecting 400pg of DNA target per PCR. The maize, soya and sugar beet assays appeared to be more sensitive and could repeatedly detect at least 8pg DNA per PCR. All LOD values are based on duplicate measurements at each point of the dilution series for each assay.

Application of Assays to Processed Food Samples

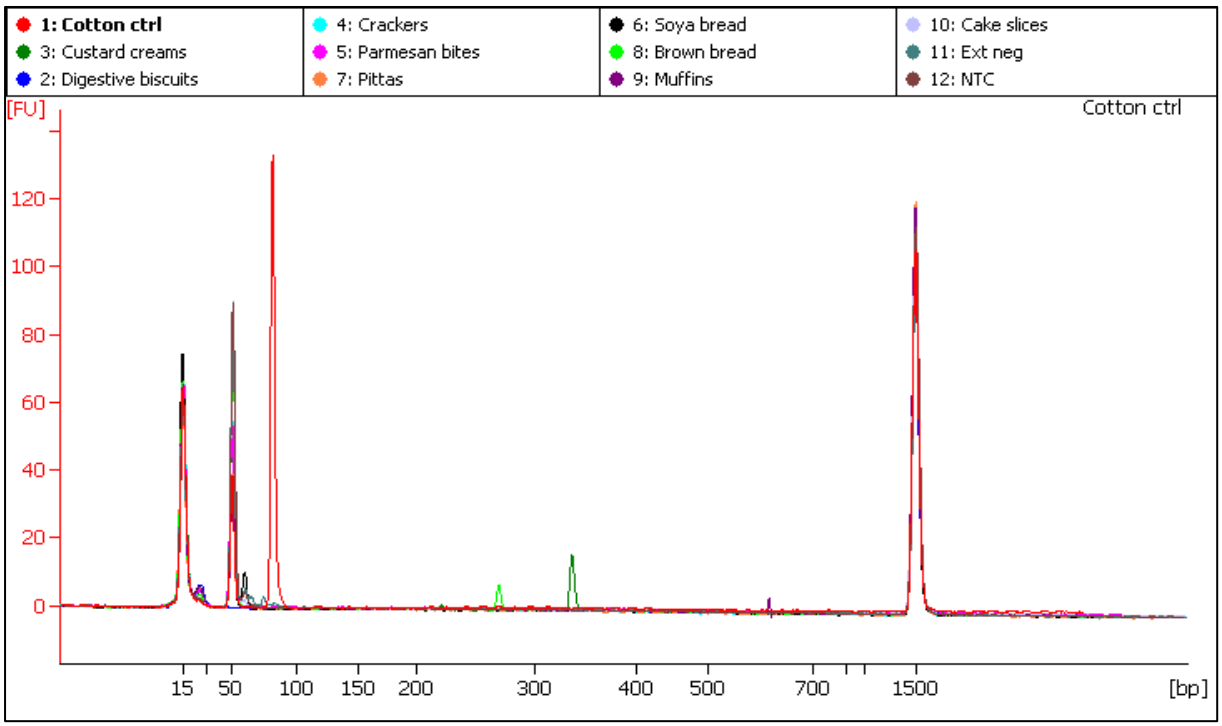
The optimised assays were applied to DNA extracted from processed food materials. DNA was extracted from the processed materials using the CTAB protocol. In general, DNA yields were over 1µg with some matrices (such as bread) extracting better than others. Each of the nine food samples detailed in Table 2 was subjected to PCR using the five optimised assays for the detection of cotton, lupin, maize, soya and sugar beet. Table 6 details the species that were detected in each food samples. Each species was detected in duplicate PCR wells per DNA extract. No false positives were observed. Cotton and sugar beet were not detected in any of the materials, but produced amplification products for the positive controls.

Table 6 – Detection of Species in Processed Food Products

Food	Relevant Species Listed in Ingredients/Allergen List	Species Detected
Trufree digestive biscuits	Maize starch, maize flour, soya flour, soya bran	Maize, Soya
Trufree custard creams	Maize starch, soya flour. May contain lupin	Lupin, Maize, Soya
Tuc crackers	n/a	None detected
Zest cheese bites parmesan	May contain soya and lupin	Lupin
Burgen soya and linseed brown bread	Soya flour, kibbled soya	Soya
Waitrose gluten free pittas	Maize flour	Maize
Genius brown bread	Maize starch, sugar beet fibre	None detected
Chocolate muffins	Emulsifier Soya lecithin[1]	None detected
Gluten free date & walnut slices	Maize flour	Maize

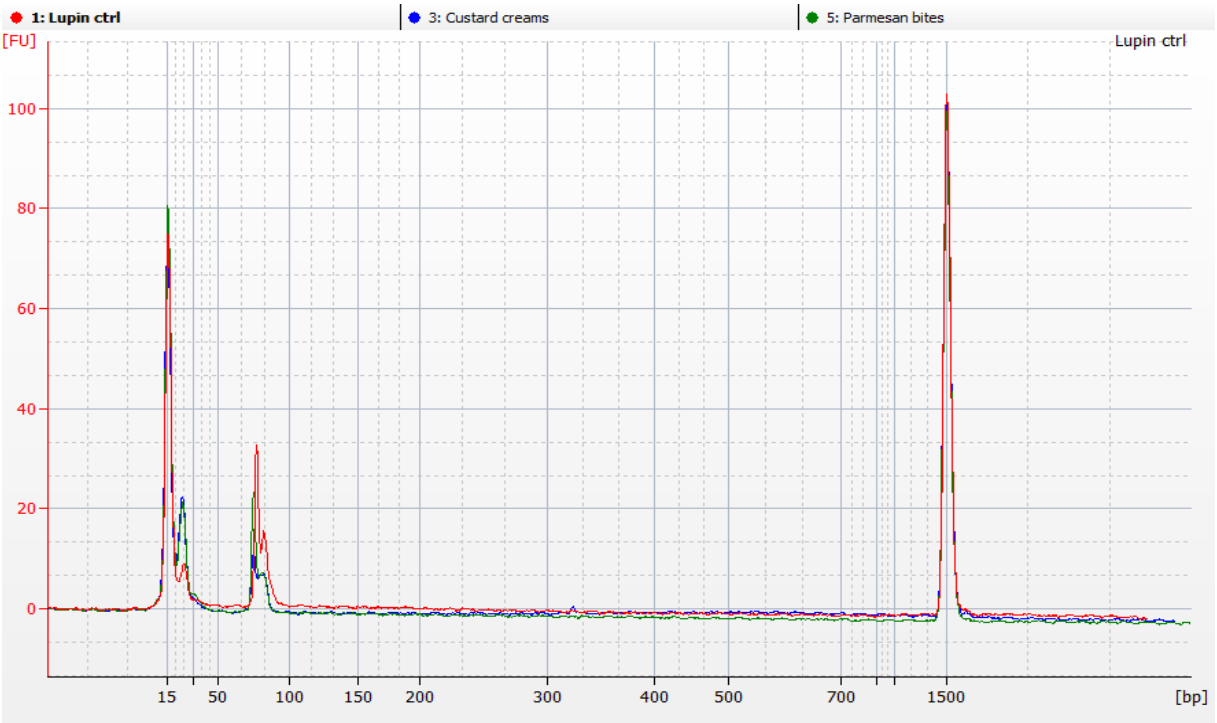
1 Soya lecithin (a mixture of phospholipids) was used as an emulsifier in the Chocolate muffins food sample

Figure 7 - Electropherogram Image for the Cotton Assay



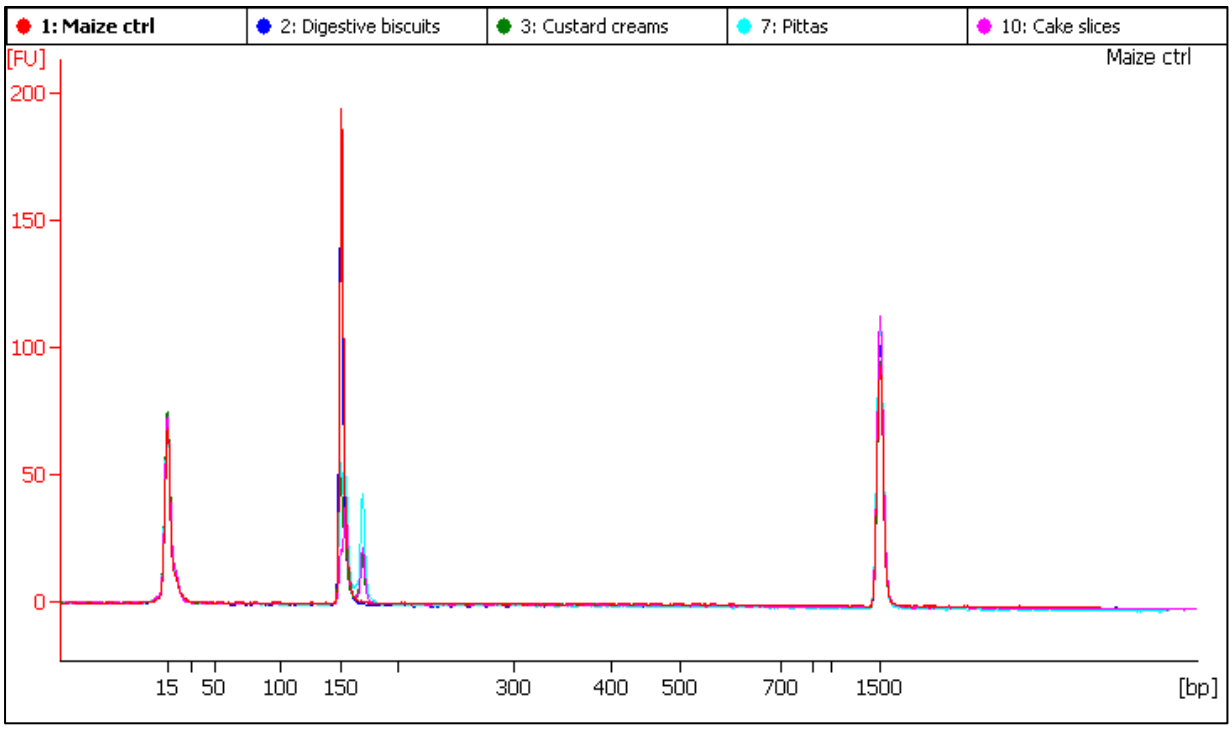
Amplicon size (bp) is represented on the x-axis and relative fluorescent units (FU) on the y-axis. A successful run is characterised by the presence of the molecular markers at 15 and 1500bp, as shown. The target 82bp amplicon is only detected in the cotton control and not in any other sample (note the ~50mers indicative of primer dimerization).

Figure 8 - Electropherogram Image for the Lupin Assay



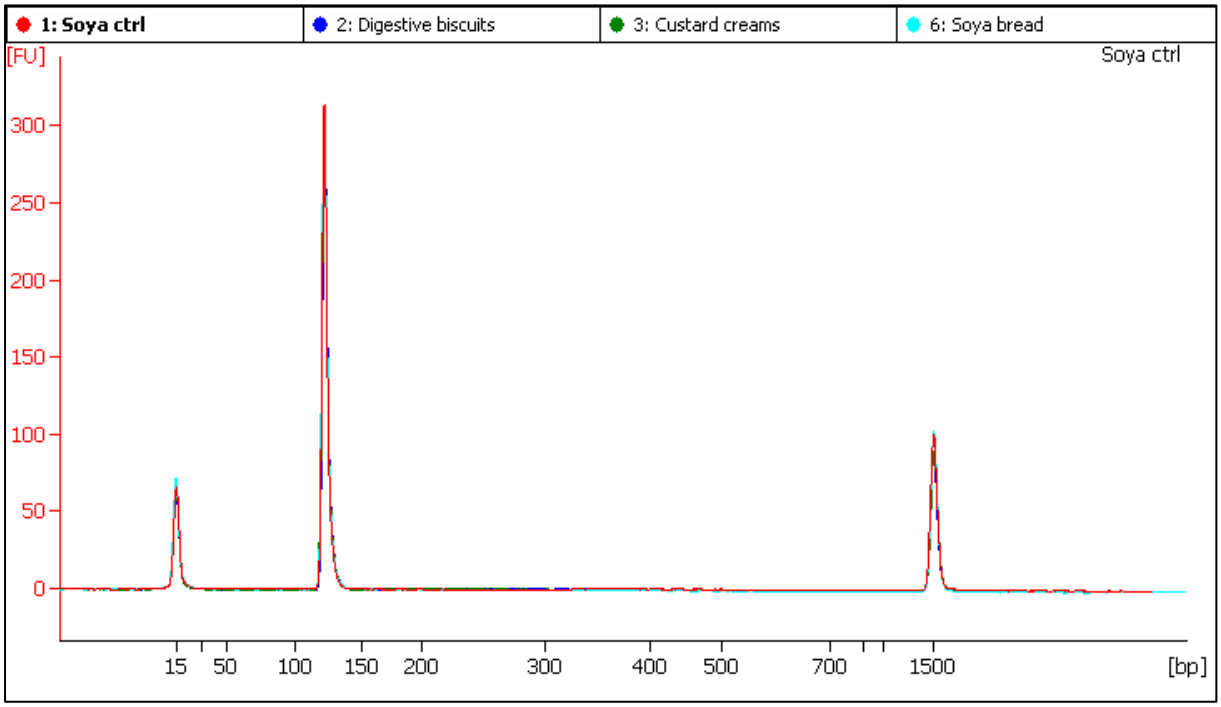
Amplicon size (bp) is represented on the x-axis and relative fluorescent units (FU) on the y-axis. A successful run is characterised by the presence of the molecular markers at 15 and 1500bp, as shown. The fluorescence threshold was lowered to 10FU on analysis to incorporate the positives that would have been previously disregarded owing to the inefficient performance of the assay. The lupin-specific amplicon at 76bp can be observed in the positive control, custard creams and parmesan bites.

Figure 9 - Electropherogram Image for the Maize Assay



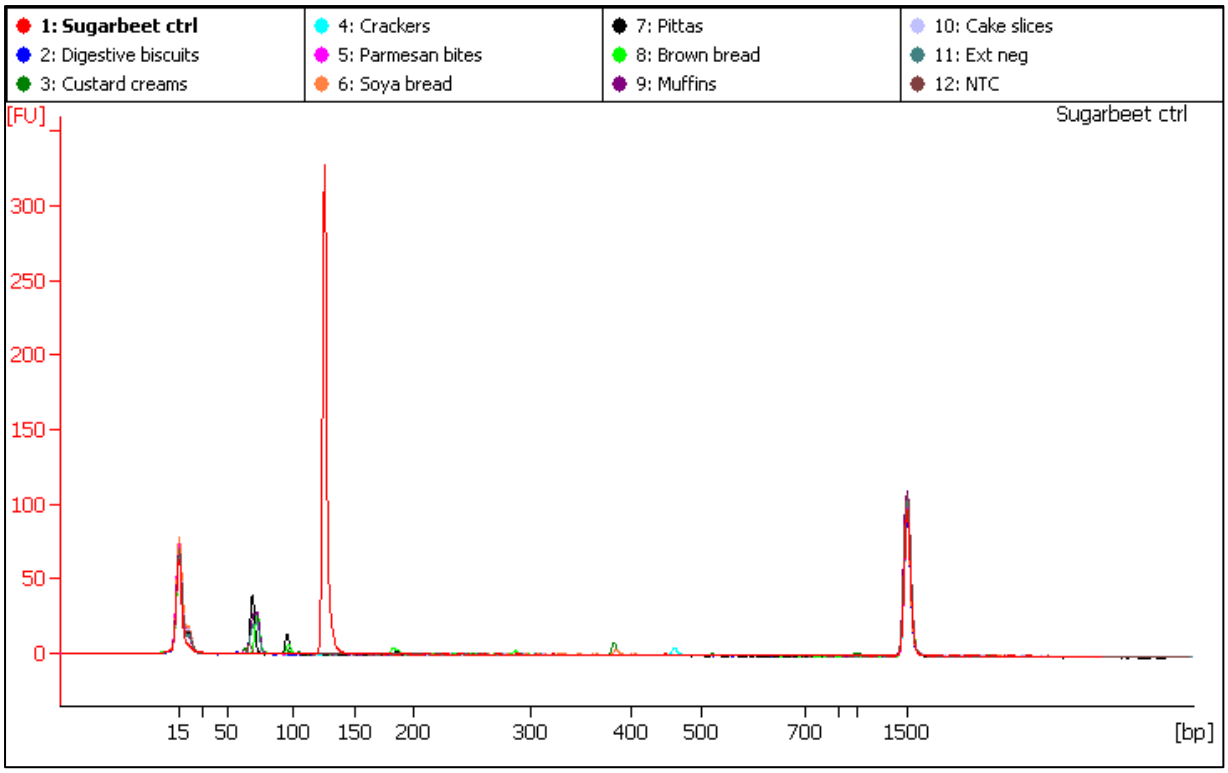
Amplicon size (bp) is represented on the x-axis and relative fluorescent units (FU) on the y-axis. A successful run is characterised by the presence of the molecular markers at 15 and 1500bp, as shown. A 151bp amplicon representative of maize being detected in the positive control can be observed in figure 9. In addition, the amplicon can also be seen in the sample wells for digestive biscuits, custard creams, pittas and cake slices. The maize amplicon was not detected in any other samples.

Figure 10 - Electropherogram Image for the Soya Assay



Amplicon size (bp) is represented on the x-axis and relative fluorescent units (FU) on the y-axis. A successful run is characterised by the presence of the molecular markers at 15 and 1500bp, as shown. The 123bp amplicon indicative of the presence of soya was observed in the positive control, digestive biscuits, custard creams and soya bread. Note that soya DNA was not detected in the sample of "chocolate muffins" – this was not unexpected as the listed ingredient of soya lecithin is an emulsifying agent consisting of a mixture of phospholipids so the sample did not necessarily have any soya DNA present in it.

Figure 11 - Electropherogram Image for the Sugar Beet Assay



Amplicon size (bp) is represented on the x-axis and relative fluorescent units (FU) on the y-axis, and the success of the run is characterised by the presence of the molecular markers at 15 and 1500bp. The sugar beet target amplicon at 125bp was only detected in the sugar beet control (note that ~60mers are present that suggest primer dimerization). These results were reproducible across duplicate PCR runs.

Discussion

DNA Extractions – In-house Validated CTAB Method

The CTAB method for DNA extraction proved to be effective in isolating nucleic acids from a range of matrices, including CRM flours, fresh plant materials and processed food. The principles of the method are described by Murray and Thompson[10], where CTAB can be used to separate plant DNA from inhibitory contaminants such as polysaccharides and tannins. Matrices that gave high DNA yields included fresh plant materials such as cauliflower, celery and okra. In these instances, cell-rich leaf material containing an ample source of nucleic acid was ground and extracted from. It is likely that the more processed food samples contained large amounts of other components such as carbohydrates, fats and proteins in relation to extractable nucleic acids, which may explain why these materials yielded less DNA

following extraction. However, it should be noted that all food samples yielded enough DNA that was of sufficient quantity and quality to be used in downstream PCR.

In light of the low A260/A230 purity values observed for some samples, namely lupin and soya, a dilution series was constructed for each assay to test for PCR inhibition in the positive control materials. Such values may have been the result of inorganic salt carryover following DNA extraction, which may interfere with PCR. However, no significant difference in amplicon detection was noted at different concentrations of template. The DNA extracts were therefore deemed as fit-for-purpose for use in the study although it is recommended that further work be conducted regarding DNA purification on those extracts that showed low A260/A230 and A260/A280 ratios to ensure that the amplifiable template is of the highest possible quality.

Cross-Reactivity Studies

Each assay was subject to pair-wise combinations with all other positive control materials (cotton, lupin, maize, soya, sugar beet, aubergine, cauliflower, celery, mustard, okra, potato, oilseed rape and rice DNA) to assess potential levels of cross reactivity. Significant cross-reactivity would occur when an assay amplified a product of a similar size (±10bp) to the positive control material which was detected by the Agilent Bioanalyzer over the set fluorescence threshold of 20FU, but in no instances was any significant cross reactivity observed.

It was noted that the cotton and lupin assays exhibited no significant cross reactivity with any other species and any amplicons that were produced were greatly different in size to that of the positive controls (over 100bp difference). The maize, soya and sugar beet assays displayed some amplification in the oilseed rape, sugar beet and maize templates, respectively but the amplicons produced were over 10bp different in size to the positive control material. This was confirmed by follow-up experiments utilising multiple replicates for each template. These experiments demonstrated that, whilst some non-specific amplification was exhibited, the resulting amplicons were sufficiently distinguishable from those of the positive control material. Based on this observation it was decided that the assays were fit for purpose for the detection of DNA from their respective species. All five assays exhibited no significant cross reactivity and were carried forward for optimisation.

Primer Optimisation

The five assays for detection of cotton, lupin, maize, soya and sugar beet were optimised to be run under identical PCR cycling conditions. This was conducted so that all assays could be run in the same batch to facilitate simultaneous detection of each species in the same food sample. The PCR variables in these experiments were primer concentration (µM) and annealing temperature (°C).

The assays were subjected to PCR with different combinations of primer concentrations (0.25, 0.50, 0.75 and 1.00µM) and annealing temperatures (56, 58, 60 and 62°C), using the

relevant positive control material as the DNA template. The optimal conditions were chosen based on whether each assay was successful in amplifying the correct size amplicon from the positive control material, with minimal or no non-specific amplification. Amplification was deemed as being most efficient when a clear peak was observed at the correct size on the electropherogram following electrophoresis, which was over the fluorescence threshold as assigned by the Agilent 2100 Expert software. Based on the results obtained it was decided that the optimal PCR conditions for all assays included a primer concentration of 0.25µM and an annealing temperature of 62°C.

It is worth reiterating that the lupin assay, whilst effective in amplifying the DNA target, suffered from poor amplification efficiency. Further work could include additional optimisation or even minor re-design of this assay to enhance amplification of the DNA target and add further value to the principles of the present study.

Determination of the LOD of each Assay

The LOD for each assay was expressed in amount of DNA (pg) per PCR. These units of expression were chosen because the assays in question included primers that were based on DNA sequences from both nuclear and chloroplast gene targets. This would have made any calculations of copy numbers complicated and incomparable so it was decided to characterise the LOD using units of expression that could be applied to all of the assays.

Characterisation of the LOD of a qualitative end-point PCR assay can be problematic owing to amplicon analyses being somewhat subjective, especially when compared with real-time PCR. When characterising the LOD for each of the PCR assays in the present study a positive result for amplification was confirmed when the Bioanalyzer detected a peak at the appropriate amplicon size that was over the assigned fluorescence threshold of 20FU. Amplification must also occur in both of the two replicates for the corresponding DNA amount in pg/PCR to be considered repeatable in amplifying the correct product from the relevant positive control. In order to further characterise the LOD more accurately additional experiments should be undertaken for each assay using a higher level of replication. However, for the purposes of providing proof-of-principle in the present study it was decided that a replication level of two was sufficient to give an indication of where the LOD is likely to be for each assay.

Application of Assays to Processed Food Samples

The optimised assays were applied to a range of processed food samples to demonstrate their applicability in the detection of each species by PCR. Proof-of-principle for the method was demonstrated, along with how this type of system could be applied to a range of different ingredients and sample types. The basis of the present study was to design a lab-on-a-chip system where a particular PCR assay could be applied to a number of samples and the PCR products run on the Agilent 2100 Bioanalyzer to confirm the presence (or absence) of a particular species. Five assays were run under the same PCR cycling conditions on a 96-well plate and each assigned a lab chip for amplicon detection. The entire process was complete

within a few hours, thus highlighting the usefulness of this system as a rapid and simple molecular tool for the detection of a particular species within a food product. It is envisaged that this approach could be rapidly applied and deployed as a screening assay by Public Analyst and related laboratories involved in testing for food authenticity. The assays for lupin, maize and soya were all successful in detecting their respective ingredients in the processed food samples. This demonstrates potential for the lupin assay in particular, which appeared to have a compromised amplification efficiency compared to the other four assays. However, when the assay was applied to a sample (Parmesan Cheese Bites) with a suspected "trace" amount of the target species included in the ingredients list, it was successful in repeatedly detecting the assigned DNA target.

The two species of cotton and sugar beet were not detected in any of the processed food materials but produced strong responses in the presence of the respective positive controls. This was not surprising as these were not listed on the ingredients list of the food samples surveyed. It is worth noting that when used in food preparation cotton seed is added as cotton seed oil (sometimes included in "vegetable oil"). Oils are notoriously difficult to extract DNA from and any nucleic acid present is likely to be destroyed during processing of the crude extract. Therefore, the presence of such ingredients in a processed food may be missed if limited amounts of DNA are available.

The detection of ingredients included in the "may contain" section of the product ingredient list (which can indicate that allergens are present at trace levels) demonstrates the applicability of the assays to detect certain species at low levels. All of the assays had a limit of detection of at least 400pg of DNA per PCR suggesting that the assays are fit for purpose for the detection of trace ingredients in food. This has implications for the use of similar DNA-based systems for the detection of allergens and other food ingredients in the future.

Conclusions

PCR assays for the detection of cotton, lupin, maize, soya and sugar beet were taken from a range of published literature and in-house assay design and then subject to further optimisation to facilitate their simultaneous application for the detection of the five target species from DNA extracted from food samples. The primers were tested by end-point PCR and amplicons were analysed by capillary electrophoresis using the Agilent 2100 Bioanalyzer. Assay development involved testing the specificity and sensitivity of the primers, optimising the reaction conditions and then applying the assays to DNA extracted from processed food materials. Each of the five assays was successful in amplifying the specific DNA target in the relevant positive control material using a common PCR cycling programme. The assays for lupin, soya and maize were all successful in repeatedly amplifying the appropriate DNA target from many of the processed food samples. Whilst successful in amplifying DNA from the positive control materials, the assays for cotton and sugar beet assays only produced detectable amplification when in the presence of the relevant positive control material, and produced no false positive when tested against the processed food materials. The present study has therefore been successful in developing a simple, singleplex PCR lab-on-a-chip approach for the detection of a range of ingredients in foods,

the principles of which can be applied to various fields within the area of food authenticity testing.

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References

1 http://www.food.gov.uk/enforcement/monitoring/horse-meat/timeline-horsemeat/; (Accessed 6th November 2013)

2 http://www.bbc.co.uk/news/uk-21681627; Horsemeat scandal: Public quizzed on contamination, 6 March 2013 (Accessed 6th November 2013)

3 Elliott Review into the Integrity and Assurance of Food Supply Networks – Final Report: A National Food Crime Prevention Framework (July 2014) (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/350726/elliot-review-final-report-july2014.pdf)

4 Moore J, Spink J, and Lipp M, (2012), Development and Application of a Database of Food Ingredient Fraud and Economically Motivated Adulteration from 1980 to 2010, Journal of Food Science, **77**(4), 118-126

5 Mafra I, Ferreira I, Oliveira M, (2008), Food Authentication by PCR-Based Methods, European Food Research and Technology, **227**, 649-665

6 Słowianek M, Majak I, (2011), Methods of Allergen Detection based on DNA Analysis, Biotechnology and Food Science, **75**(2), 39-44

7 http://infoallergy.com/Tools-Extras/foodallergycampaign/statistics/ (Accessed 5th November 2013)

8 Annex IIIa to Directive 2000/13/EC, Commission Directive 2007/68/EC, 27th November 2007 to be superseded by Annex II to Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the Provision of Food Information to Consumers, OJ L304/18 22.11.2011 coming into force 13 December 2014 with regard to allergens

9 Primer - Basic Local Alignment Search Tool (Primer-BLAST) U.S. National Library of Medicine (NLM), National Center for Biotechnology Information (NCBI) at the

National Institutes of Health (NIH), 2010, http://blast.ncbi.nlm.nih.gov/Blast.cgi (Accessed November 2013)

10 Murray M, Thompson W, (1980), Rapid Isolation of High Molecular Weight Plant DNA, Nucleic Acids Research, **8**(19), 4321-4325

11 JRC Reference Reports: Compendium of Reference Methods for GMO Analysis, 2010, EUR 24526 EN

12 Standard Operating Procedure for the Simultaneous Detection of General DNA Targets Associated with the Presence of Genetically Modified Soya and Maize, Food Standards Agency project G03022, GMO multiplex screen trial for Public Analysts July/August 2008 - Version 1

13 Event-Specific Method for the Quantification of Cotton Line "LLCotton25" Using Real-time PCR, Protocol, 14th March 2007, CRL-GMFF: Protocol LLCotton25

14 Event specific Method for the Quantification of Sugar Beet line H7-1 using real time PCR, Validation Report and Protocol, 18th January 2006, EUR 22134 EN